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# Krüppel-like Factor 4 Inhibits Tumorigenic Progression and Metastasis in a Mouse Model of Breast Cancer[1,2]

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## Abstract

Krüppel-like factor 4 (KLF4) is a zinc finger transcription factor that functions as an oncogene or tumor suppressor in a highly tissue-specific cell-dependent manner. However, its precise role in breast cancer and metastasis remains unclear. Here, we show that transient adenoviral expression of KLF4 in the 4T1 orthotopic mammary cancer model significantly attenuated primary tumor growth as well as micrometastases to the lungs and liver. These results can be attributed, in part, to decreased proliferation and increased apoptosis. Further supporting a tumor-suppressive role for KLF4 in the breast, we found that KLF4 expression is lost in a mouse model of HER2/NEU/ERBB2–positive breast cancer. To determine whether enforced KLF4 expression could alter tumor latency in these mice, we used a doxycycline-inducible expression model in the context of the MMTV-Neu transgene. Surprisingly, tumors that developed in this model also lost KLF4 expression, suggesting negative selection for sustained expression. We have previously reported that KLF4 inhibits epithelial-to-mesenchymal transition (EMT), a preliminary step in metastatic progression. Overexpression of KLF4 in 4T1 cells led to a significant reduction in the expression of Snail, a key mediator of EMT and metastasis. Conversely, KLF4 silencing increased Snail expression in the non-transformed MCF-10A cell line. Collectively, these data demonstrate the first functional, *in vivo* evidence for KLF4 as a tumor suppressor in breast cancer cells. Furthermore, our findings suggest an inhibitory role for KLF4 during breast cancer metastases that functions, in part, through repression of Snail.

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Abbreviations: KLF4, Krüppel-like factor 4; EMT, epithelial-to-mesenchymal transition; MMTV, mouse mammary tumor virus; rtTA, reverse tetracycline transactivator; MTB, tetracycline-inducible transgenic mouse line; qRT-PCR, quantitative real-time polymerase chain reaction; TBP, TATA-box binding protein; shKLF4, stable short-hairpin knockdown KLF4 cells; shNS, stable short-hairpin nonspecific knockdown control cells; AdKLF4, adenovirus overexpressing KLF4; AdGFP, empty vector adenovirus control; BrdU, bromodeoxyuridine

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## Introduction

Krüppel-like factor 4 (KLF4) is a zinc-finger transcription factor that regulates a multitude of processes in normal tissue including proliferation, differentiation, apoptosis, tissue homeostasis, and self-renewal [1–5], to name a few. KLF4 can also have both tumor-suppressive and oncogenic roles in cancer. KLF4 was first identified as a tumor suppressor, owing to frequent loss of expression in colon, esophageal, gastric, bladder, prostate, and lung cancers [6–11]. Subsequent studies, however, have suggested a role for KLF4 as an oncogene in other tissues, including breast [12,13]. Paradoxically, KLF4 was most recently found to suppress estrogen-dependent breast cancer cell growth, and its expression is inversely correlated with increasing tumor stage and grade [14], further confounding its role in this disease.

In human mammary epithelial cells, KLF4 is a transcriptional activator of E-cadherin and suppressor of epithelial-to-mesenchymal transition (EMT) [15]. This process, whereby cells acquire a fibroblast-like morphology and altered molecular signature associated with reduced cellular adhesion and increased motility, is critical not only during development but may also be involved in cancer progression [16,17]. While controversy still persists over the existence of EMT during tumorigenesis and metastasis, compelling evidence supporting its relevance in human carcinomas, including breast, prostate, melanoma, and others, has been reported (see review of Blick et al. [18]). A hallmark of EMT is the loss of E-cadherin expression, which is transcriptionally suppressed by Snail [19]. In breast cancer, both loss of E-cadherin and increased Snail expression are predictive of poor prognosis [20]. Furthermore, Snail correlates with increasing tumor grade, lymph node status, and metastasis [21].

Disease recurrence and distant metastases are the leading cause of death in breast cancer patients. One subtype of breast cancer that is highly metastatic involves the amplification and overexpression of the *HER2/Neu* gene in 15% to 30% of all breast cancer cases. Elevated expression of this receptor tyrosine kinase is associated with a higher risk of recurrence because tumors that are initially responsive to trastuzumab (Herceptin) often become resistant. One mechanism by which HER2/*Neu*–dependent tumors can recur is through upregulation of Snail [22]. However, the mechanisms by which Snail is induced are not well understood. Here, we demonstrate that forced expression of KLF4 in the clinically relevant, orthotopic 4T1 xenograft model inhibits primary tumor growth and metastases. We also show that KLF4 expression is suppressed in a mouse model of HER2/*Neu*–induced tumorigenesis. Furthermore, *in vitro* analyses revealed that KLF4 is able to suppress Snail expression, suggesting a mechanism whereby loss of KLF4 leads to increased breast cancer metastasis.

## Materials and Methods

### *Transgenic Mice*

All mice were housed in microisolator-plus units under pathogen-free conditions in a 12-hour light-dark cycle. Food and water were provided *ad libitum*. The NeuN mice (FVB/N-TgN(MMTV-neu)202Mul), containing the rat proto-oncogene c-*neu* transgene [23], and the NeuT mice (FVB/N-Tg(MMTV-Erbb2)$NK_1$Mul/J), containing the activated c-*neu* transgene [24], both targeted to mammary epithelium by the MMTV-LTR promoter, were purchased from Jackson Laboratories (Bar Harbor, ME). The TRE-KLF4 and MMTV-*rt*TA (MTB) mice, both on the FVB/N genetic background, have been previously characterized [3,25]. Mice were killed once initial tumors had reached 1.5 cm by caliper measurement. All animal studies were approved by the Case Western Reserve University Institutional Animal Care and Use Committee.

### *Microarray and Expression Analyses*

Microarray analyses comparing age-matched wild-type glands (AMWT), hyperplastic tissue adjacent to the tumor, and NeuN tumors has been described in detail [26]. Quantitative real-time polymerase chain reaction (qRT-PCR) was performed on RNA harvested from tissue and cells as previously described [15,26] using mouse and human gene–specific TaqMan assays (Applied Biosystems, Carlsbad, CA). RNA levels were normalized against mouse *Gapdh*, mouse *β-actin*, or human TATA box binding protein (*TBP*) as indicated.

### *Cell Culture and Generation of 4T1-Luciferase Cells*

The MCF-10A and 4T1 cells lines were purchased from ATCC (Manassas, VA). Generation and characterization of MCF-10A cells with stable knockdown of KLF4 (shKLF4) have been reported [15]. The MCF-10A and 4T1 progression series were obtained from Dr Fred Miller at Karmanos Cancer Institute. All MCF-10A lines, including the MCF-10AT1K, MCF-10CA1h, and MCF-10CA1a, were cultured in complete medium [27]. All 4T1 lines, including the 67NR and the 4T07, were cultured in Dulbecco modified Eagle medium (Mediatech, Inc, Manassas, VA) supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin.

4T1-luciferase–expressing cells (4T1-luc) were generated by subcloning the complete gene from the Luciferase T7 Control DNA (Promega, Madison, WI), as a *Bam*HI/*Sac*I fragment into pBlueLZRS-2. Subsequent digestion yielded the luciferase containing *Bam*H1/*Sfi*I fragment, which was inserted into the LZBOB-Hygro retroviral expression vector. Virus was generated using the Phoenix packaging cells as described [28], with modifications. (Vectors and Phoenix cells were kind gifts from Keith R. Johnson, University of Nebraska Medical Center). Briefly, transfected Phoenix cells were cultured for 48 hours at 37°C before virus-containing medium was harvested. 4T1 cells were plated overnight at a low density and infected the next day with 0.45-μm filtered virus-containing medium, supplemented with 4 mg/ml polybrene (Sigma, St Louis, MO). The medium was changed after overnight incubation at 32°C. Infected 4T1 cells were cultured to 80% confluency and selected in 100 μg/ml hygromycin (Invitrogen, Carlsbad, CA).

### *Transwell Migration and Invasion Assays*

Assays were performed as previously described [15]. Briefly, $5.0 \times 10^5$ cells were seeded onto transwell supports (Costar, Corning, NY) or BD Matrigel Invasion Chambers (BD Biosciences, Bedford, MA) and allowed to migrate or invade toward complete medium for the indicated times. Five (20×) fields per transwell support were counted.

### *Bromodeoxyuridine Incorporation and Fluorescence-Activated Cell Sorting Analysis*

4T1 cells were infected with empty vector adenovirus control (AdGFP) or KLF4-expressing adenovirus (AdKLF4) [15] for 72 hours before a 30-minute incubation with 10 μM bromodeoxyuridine (BrdU; Sigma-Aldrich) at 37°C. Cells were fixed and labeled with anti-BrdU (BD Biosciences) and analyzed by fluorescence-activated cell sorting (FACS) as previously described [15].

### *4T1 Orthotopic Tumor Model*

Twenty-four hours before injection, 4T1 or 4T1-luc cells were infected with AdGFP control or AdKFL4. The next day, cells were trypsinized, washed, and counted before resuspension in Dulbecco modified Eagle medium. Female BALB/c mice (Jackson Laboratories), 6 to 8 weeks old, were inoculated with $0.5 \times 10^6$ cells (50 μl) in a no. 9 mammary fat pad. Tumors were measured weekly by calipers. Mice injected with luciferase-expressing cells were anesthetized with 2% isoflurane and injected intraperitoneally with 200 μl of D-luciferin potassium salt at 12.5 mg/ml in phosphate-buffered saline 5 minutes before imaging on a Xenogen IVIS 200 scanner (Caliper Sciences, Hopkinton, MA).

### *Morphologic and Immunohistochemical Analysis*

Tissues were fixed overnight in 4% paraformaldehyde and embedded in paraffin. Five-micrometer sections were dewaxed and stained with hematoxylin and eosin. For immunostaining, cleared sections were rehydrated, and antigen retrieval was performed using 10 mM sodium citrate buffer (pH 6.0) in a pressure cooker for 20 minutes at 125°C. Endogenous peroxidases were quenched in block (Dako EnVision+ System-HRP; Dako, Carpinteria, CA) containing 15 μl/ml normal goat serum (Jackson Immunologicals, West Grove, PA), and sections were incubated overnight at 4°C with primary antibodies: anti-KLF4 (1:500, H-180; Santa Cruz Biotechnology, Santa Cruz, CA), anti-Flag (1:500, M2; Sigma), or anti–cytokeratin 5/6 (1:20; Zymed, Heidelberg, Germany). Slides were washed in Tris-buffered saline–0.05% Tween 20 and incubated with labeled polymer–horseraddish peroxidase antirabbit or antimouse (Dako) for 1 hour at room temperature. The signal was developed using 3,3′-diaminobenzidine, and slides were counterstained with hematoxylin and mounted. Images were captured using a Nikon microscope (Nikon Instruments, Inc, Melville, NY) and Zeiss AxioCam HRc imaging system (Carl Zeiss MicroImaging, LLC, Thornwood, NY).

### *Identification of Lung and Liver Metastases*

Lung metastases were identified by *in vivo* bioluminescence imaging or hematoxylin and eosin–stained sections. Six sections per lung, collected at 100-μm intervals, were evaluated. Two liver sections per mouse, separated by at least 100 μm were stained with cytokeratin 5/6 and counted to quantify tumor cell infiltrate and micrometastases.

### *Western Blot Analysis*

Cells were lysed in radioimmunoprecipitation assay buffer as previously described [15], and 50 μg of whole-cell lysates was separated on 12% SDS-PAGE. Western blots were probed with primary

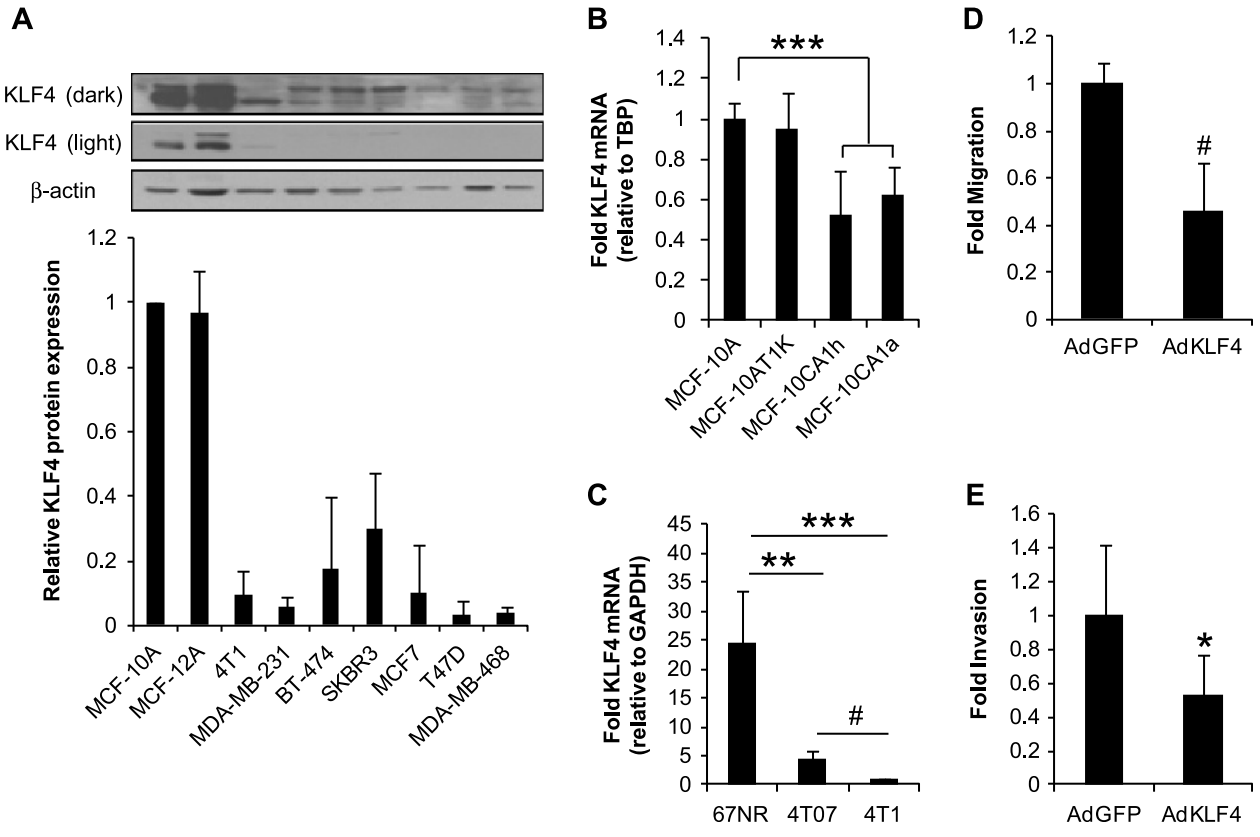


**Figure 1.** *KLF4* expression is low in breast cancer cell lines compared with nontransformed mammary epithelial cell lines and inversely correlates with metastatic progression. (A) Western blot and densitometric quantification comparing KLF4 expression among nontransformed MCF-10A and MCF-12A mammary epithelial cells and breast cancer cell lines. qRT-PCR analysis of the (B) MCF-10A and (C) 4T1 progression series reveals decreasing *KLF4* expression with increasing tumorigenicity and/or metastatic capacity. Protein or total RNA was harvested from cells at 60% to 70% confluency. (D) Transwell migration of 4T1 cells is inhibited by KLF4. (E) KLF4 inhibits 4T1 cell invasion into the Matrigel. Cells were allowed to migrate or invade, toward complete medium for 6 and 24 hours, respectively. Experiments were performed at least three times in triplicate. $^*P < .01$. $^{**}P < .001$. $^{***}P < 5.0e-4$. $^{\#}P < 1.0e-4$. Overexpression of KLF4 in cells used for migration and invasion experiments is confirmed in Figure 3*A*.

antibodies directed to KLF4 (Millipore, Billerica, MA); N-cadherin, Snail, cleaved caspase 3, and cleaved caspase 7 (Cell Signaling, Beverly, MA); E-cadherin and P120 (BD Transduction Laboratories, Bedford, MA); β-actin (Sigma); and vimentin (Santa Cruz Biotechnology). Peroxidase-conjugated secondary antibodies were from Santa Cruz Biotechnology. Blots were developed with Pierce ECL Western Blotting Substrate (Thermo Scientific, Rockford, IL). Bands were quantified using ImageJ software (Rasband, WS, US National Institutes of Health, Bethesda, MD).

### *Chromatin Immunoprecipitation*

Chromatin immunoprecipitation (ChIP) was performed as previously described [15] or using the human KLF4 ExactaChip (R&D Systems, Minneapolis, MN) following the manufacturer's protocol.

### *Statistical Analyses*

Statistical analyses were performed using two-tailed Student's *t* test, unless otherwise stated in figure legends, with $P < .05$ considered statistically significant. Error bars represent SDs.

## Results

### *KLF4 Expression Is Inversely Correlated with Metastatic Progression and Inhibits the Migration and Invasion of 4T1 Tumor Cells*

While questions exist regarding the role of KLF4 in breast cancer, Figure 1*A* reveals that KLF4 protein expression is greatly reduced in a variety of breast cancer cell lines, representing many of the breast cancer subtypes, compared with nontransformed MCF-10A and MCF-12A mammary epithelial cells. These data further support a recent report indicating that KLF4 expression is downregulated in primary human breast tumors compared with normal breast [14]. Previous work in our laboratory demonstrated that forced expression of KLF4 was able to inhibit migration and invasion of the metastatic MDA-MB-231 breast cancer cell line [15]; however, the relationship between KLF4 expression and metastasis remains unclear. We examined KLF4 expression in two cell line series that have been widely used to study the molecular events during breast cancer development and metastasis. The MCF-10A series is composed of at least four isogenic lines, representing normal breast epithelium (MCF-10A), benign cells (MCF-10AT1K), carcinoma *in situ* (MCF-10ACA1h), and invasive carcinoma (MCF-10ACA1a), the latter of which forms metastases to the lungs [29,30]. The 4T1 series consists of several lines that were isolated from a single spontaneously arising BALB/c mouse mammary tumor [31]. When orthotopically injected into the mammary fat pad, all lines form primary tumors with different metastatic propensities. We used the 4T1 line, which is highly metastatic to multiple sites; the 4T07 line, which forms micrometastases at several sites; and the 67NR line, which is nonmetastatic. To determine whether KLF4 expression is reduced with increasing metastatic potential, we performed qRT-PCR of both series. *KLF4* expression decreases with increasing metastatic capacity in the MCF-10A series (Figure 1*B*). Likewise, *KLF4* is lowest in the most metastatic line of the 4T1 series and is elevated in the less metastatic sibling lines (Figure 1*C*). We have previously shown that loss of KLF4 results in increased migration of MCF-10A cells [15]. To determine whether KLF4 overexpression has the opposing effect in the aggressive 4T1 cell line, these cells were transduced with an adenoviral KLF4 overexpression vector (AdKLF4). KLF4 overexpresssion caused a significant reduction in both migration (Figure 1*D*) and invasion (Figure 1*E*) when compared with adenovirus empty vector control (AdGFP) cells. Thus, reduced *KLF4* expression during metastatic progression and the ability of KLF4 to reduce the migratory and invasive capacities of mammary cancer cells suggest a role for KLF4 in preventing breast cancer metastasis.

### *KLF4 Alters the Growth of 4T1 Cells by Inhibiting Proliferation and Increasing Apoptosis*

KLF4 is an established regulator of proliferation through transcriptional control of multiple cell cycle factors [32]. Bright-field microscopy revealed decreased cell number in KLF4-overexpressing 4T1 cells when compared with AdGFP-infected controls (data not shown).

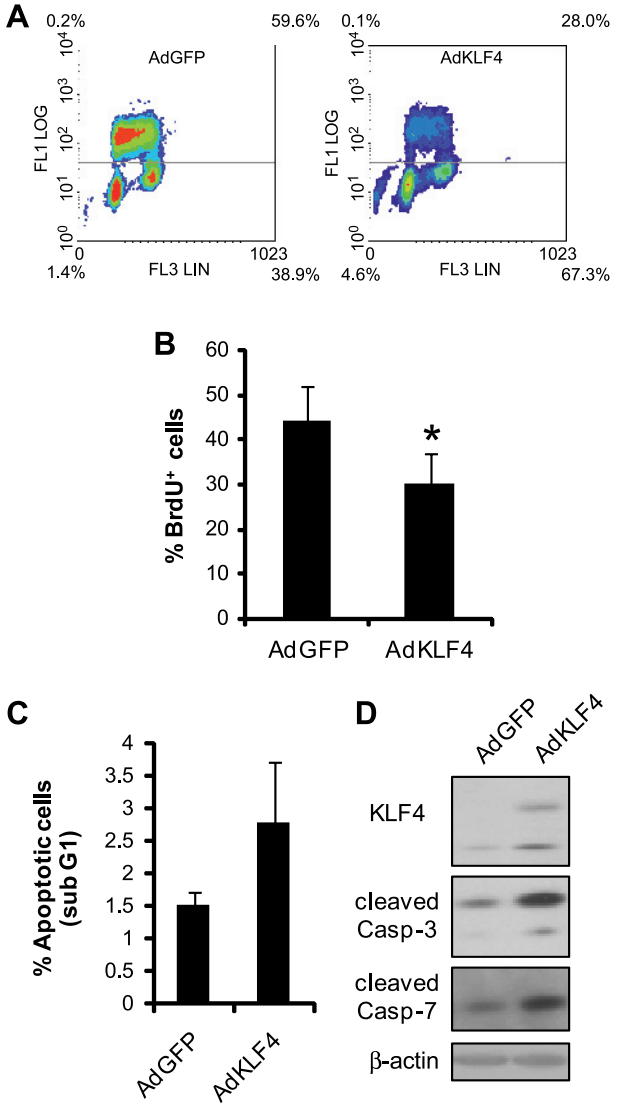


**Figure 2.** KLF4 overexpresssion inhibits growth of 4T1 cells. (A) Representative density plots from FACS analysis of 4T1 cells infected with either AdGFP or AdKLF4 for 72 hours. Cells in the upper right quadrant are positive for BrdU incorporation. Cells in the bottom left quadrant represent cells in the sub-$G_1$ phase. (B) Quantitation of BrdU incorporation (*$P < .02$) and (C) sub-$G_1$ population ($P = .08$). (D) Representative Western blot showing increased levels of cleaved caspase 3 and cleaved caspase 7 in AdKLF4-4T1 cells compared with AdGFP-4T1 control cells. Experiments were performed at least three times in triplicate.

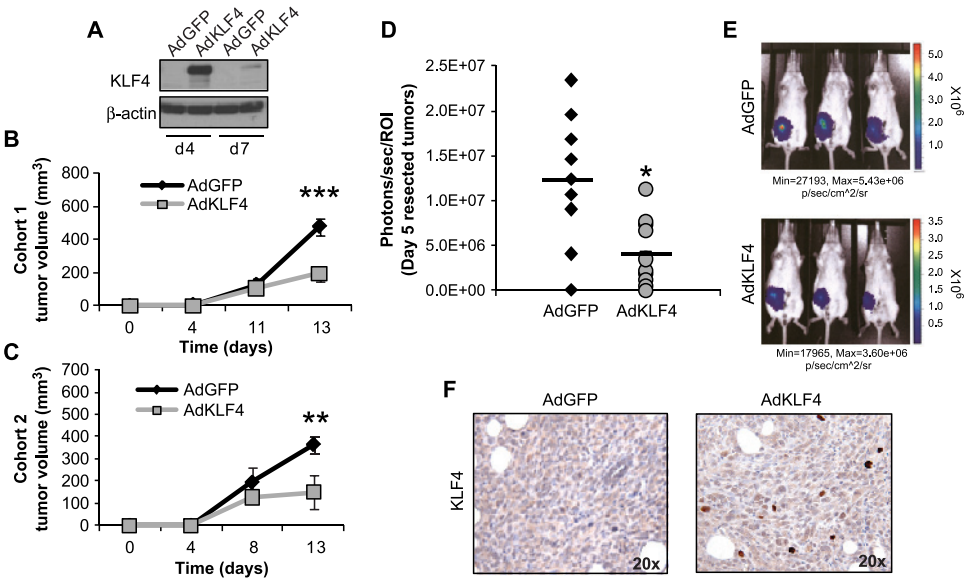


**Figure 3.** KLF4 inhibits primary tumor growth of 4T1 cells. (A) Western blot shows maintained (*in vitro*) expression of virally transduced KLF4 in 4T1 cells at days 4 and 7 after infection. (B) 4T1 ($n = 5$ per group) and (C) 4T1-luc ($n = 4$ per group) tumors expressing AdKLF4 have reduced volume compared with AdGFP control tumors. Tumors were measured using calipers at various intervals during a 2-week period. (D) AdKLF4 significantly reduces primary tumor formation determined by whole animal bioluminescence scanning at day 5 after injection (AdGFP, $n = 9$; AdKLF4, $n = 10$). Luminescence is expressed as photons/second per region of interest. (E) Bioluminescent images of three representative animals from each group in D. The relationship between color and light intensity in arbitrary units is given by the color bar to the right of each group. (F) Immunohistochemical staining of primary AdGFP-4T1 and AdKLF4-4T1 mammary tumors dissected at day 5 after injection, using anti-flag to detect exogenous KLF4. $^*P < .01$. $^{**}P < .005$. $^{***}P < 5.0e - 6$.

FACS analyses of BrdU incorporation were used to determine whether KLF4 overexpression resulted in decreased proliferation. The percentage of BrdU-positive cells was reduced in the AdKLF4-transduced cells compared with the AdGFP control cells (Figure 2, *A* and *B*). KLF4 also induces apoptosis in a variety of cancer cells including lymphoma [33], gastric [7], bladder [8], and colon [34] cancer cells. Hence, we determined whether forced expression of KLF4 also altered apoptosis in the 4T1 cells. Propidium iodide staining and cell cycle analysis indicated a trend toward an increase in the sub-$G_1$ population of the AdKLF4 cells (Figure 2, *A* and *C*), suggesting an increase in the amount of cell death. Western blot analyses further revealed an increase in cleaved caspase 3 and cleaved caspase 7 (Figures 2*D* and W1*A*), indicating that KLF4 does indeed increase apoptosis. These data demonstrate that, in addition to regulation of metastasis, KLF4 may also affect primary tumor growth of breast cancer cells by decreasing proliferation and increasing apoptosis.

### *Transient Overexpression of KLF4 Inhibits Primary Mammary Tumor Growth and Decreases Lung and Liver Micrometastases*

We next evaluated the ability of KLF4 to regulate tumor growth and progression using a xenograft model and transient overexpression of KLF4. Orthotopic injection of 4T1 cells into immunocompetent syngeneic mice, resulting in primary tumor formation and metastases, is a clinically relevant model of spontaneous breast cancer closely resembling many of the processes observed in the human disease [35]. 4T1 cells were transduced with either AdGFP or AdKLF4 and subsequently injected into the mammary fat pad of 6- to 8-week-old female BALB/c mice. An aliquot of the transduced cells was also replated and analyzed for elevated KLF4 expression, which was confirmed for up to 7 days after infection (Figure 3*A*). A 5-day time course of AdKLF4 protein expression in 4T1 cells is shown in Figure W1*A*. As shown in Figure 3*B*, the growth of primary tumors arising from AdKLF4-transduced 4T1 cells was reduced at day 13, compared with control AdGFP-4T1 tumors. In a separate experimental cohort using luciferase-expressing 4T1 cells (4T1-luc), a similar reduction in tumor volume was observed in the AdKLF4 group compared with controls (Figure 3*C*). Neither AdGFP tumors nor AdKLF4 tumors were palpable until at least 8 days after injection; however, *in vivo* bioluminescence imaging revealed a decrease in the AdKLF4 tumor size compared with the AdGFP controls at 5 days after injection (Figure 3, *D* and *E*). For mice imaged at 5 days, tumors were resected from half of the animals in each group, and KLF4 expression was confirmed in tumor sections using a Flag antibody that recognizes exogenous, adenovirally expressed, flag-KLF4 (Figure 3*F*). Five days after implantation, the percentage of strongly expressing cells is greatly reduced compared with postinfection day 1, but similar to the percentage seen in day 5 cultures, *in vitro* (Figure W1, *B* and *C*). Although KLF4-induced inhibition of growth was detectable at day 5 after implantation, no differences in proliferation (phospho-Histone H3 staining) or apoptosis (TUNEL staining) were observed at this time point (data not shown), suggesting that earlier changes in these processes must have occurred, which led to reduced tumor size at this later time point. This possibility is further supported by the greatly reduced number of cells still expressing KLF4 (Figures 3*F* and W1, *B* and *C*) and the increased expression of cleaved caspase 3 observed in the AdKLF4-infected cells compared with the AdGFP-infected controls at days 2 and 3, *in vitro* (Figure W1*A*).

4T1 tumors metastasize to various organs including lung and liver [31]. Tumor cell dissemination occurs early in this model, and early resection of primary tumors does not preclude metastatic spread

**Table 1.** Incidence of Lung and Liver Micrometastases in AdGFP-4T1 and AdKLF4-4T1 Tumor-Bearing Mice at 21 Days After Injection.

| Mice | No. of Mice with Micrometastases | |
|---|---|---|
| | Lung | Liver |
| AdGFP ($n$ = 9) | 6 | 9 |
| AdKLF4 ($n$ = 9) | 1* | 9 |

*$P$ = .025, Fisher exact test.

[36]. On the basis of our migration and invasion studies, we hypothesized that forced expression of KLF4 early during the tumorigenic process may prevent or delay metastases. To test this, we examined the lungs and livers from AdGFP control and AdKLF4 tumor-bearing mice at 21 days after injection. On removal of the lungs, no macrometastases were visible from either control or AdKLF4 group. However, bioluminescence imaging and histologic examination of multiple lung sections revealed that more than 60% of the mice in the control AdGFP group, compared with less than 10% of AdKLF4 tumor-bearing mice, had developed lung micrometastases (Table 1 and Figure 4, *A1* and *A2*). To identify tumor cells in the liver, tissue sections were stained with anti–cytokeratin 5/6 (Figure 4, *A3*–*A6*) and quantified. While all mice had developed micrometastases at this time, AdKLF4 reduced the number of micrometastases in the liver by 40% (Figure 4*B*). Hence, transient restoration of KLF4 expression attenuates early metastatic progression in a clinically relevant mouse model of breast cancer.

### *KLF4 Expression Is Suppressed in Two Mouse Models of HER2/Neu–Positive Breast Cancer*

We previously reported transcriptome analyses of MMTV-*c-neu* (NeuN)–induced mouse mammary tumors [26]. Further evaluation of these data revealed a progressive decrease in KLF4 expression with increasing tumor progression (Figure 5*A*). Microarray data were further confirmed using qRT-PCR (Figure 5*B*). In addition, we examined the expression of *KLF4* in tumors arising in NeuT mice that express the activated rat *Neu* oncogene from the MMTV promoter [24]. Similar to mice that overexpress the wild-type *Neu* oncogene (NeuN), tumors from NeuT mice also displayed a dramatic loss of *KLF4* messenger RNA (mRNA; Figure 5*B*). To determine whether enforced expression of *KLF4* would alter MMTV-NeuT–induced tumor progression, we generated a tritransgenic model that drives inducible overexpression of KLF4 in the mammary gland (Figure 5*C*). A "tet-ON" transgenic line (MTB) expressing the reverse tetracycline transactivator (rtTA), under control of the mouse mammary tumor virus promoter (MMTV-rtTA), has been previously characterized and shown to express rtTA in the mammary glands of virgin female mice as early as 5 weeks of age [37]. Mice with a doxycycline-inducible transgene that expresses KLF4 (TRE-KLF4) [25] were crossed with the MTB mice to yield the MTB/TRE-KLF4 bitransgenics. In the presence of doxycycline, these mice displayed elevated KLF4 in the mammary epithelium compared with control littermates (Figure 5*D*). We next crossed control MTB and MTB/TRE-KLF4 mice with homozygous MMTV-NeuT mice. On weaning, all mice were continuously administered doxycycline in the water for the remainder of the experiment. As expected, control MTB/NeuT mice developed tumors with a mean tumor latency of 29 weeks. Surprisingly, forced expression of KFL4 in the MTB/TRE-KLF4/NeuT tritransgenics did not significantly alter mammary tumor latency (Figure 5*E*). However, despite increased KLF4 levels in normal mammary epithelium, when we examined *KLF4* mRNA expression in these tumors compared with the controls, there was no evidence of *KLF4* overexpression (Figure 5*F*). These data indicate that KLF4 expression is incompatible with HER2/*Neu* tumorigenesis and that cells expressing KLF4 undergo negative selection during formation of mammary tumors.

### *KLF4 Inhibits Snail Expression in Both MCF-10A Mammary Epithelial Cells and 4T1 Breast Cancer Cells*

We have previously identified KLF4 as an inhibitor of EMT in mammary epithelial cells, in part, through activation of E-cadherin gene expression [15]. In contrast to KLF4, Snail plays an important role in the induction of EMT through transcriptional suppression of E-cadherin [19]. Recently, Snail expression was found to be suppressed by KLF4 during the reprogramming of mouse fibroblasts

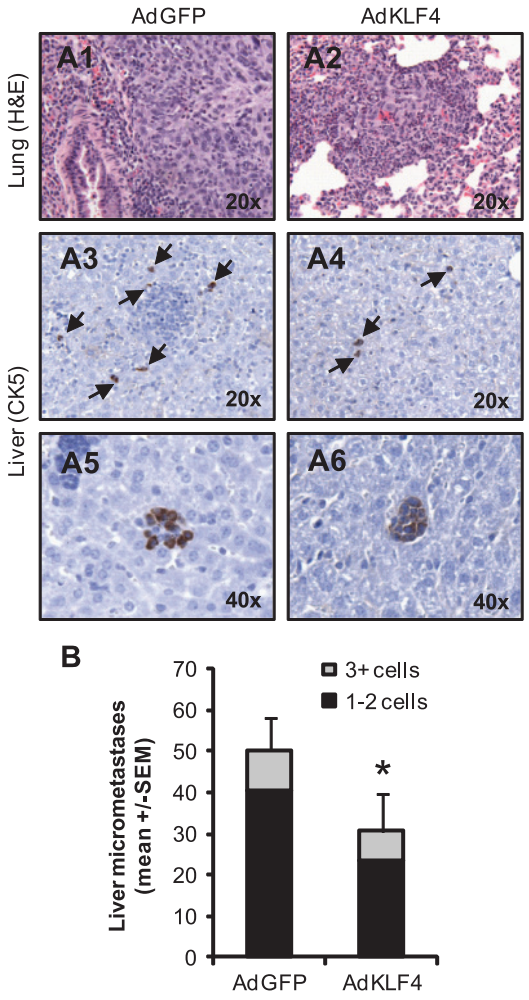


**Figure 4.** KLF4 overexpression decreases lung and liver micrometastases. (A1) Representative micrometastasis from lung of AdGFP-4T1 mouse and (A2) largest metastasis from the only AdKLF4-4T1 mouse that developed lung metastases. (A3–A6) Representative liver sections from AdGFP-4T1 mice and AdKLF4-4T1 mice stained with anti–cytokeratin 5/6 (CK5/6) to detect metastatic mammary tumor cells in the liver (arrows). (B) Quantitation of CK5/6 staining. Livers from AdKLF4-4T1 mice had a significant reduction in micrometastases compared with AdGFP-4T1 mice (Mann-Whitney test, *$P$ < .05). Both lungs and livers were harvested at day 21 after injection.

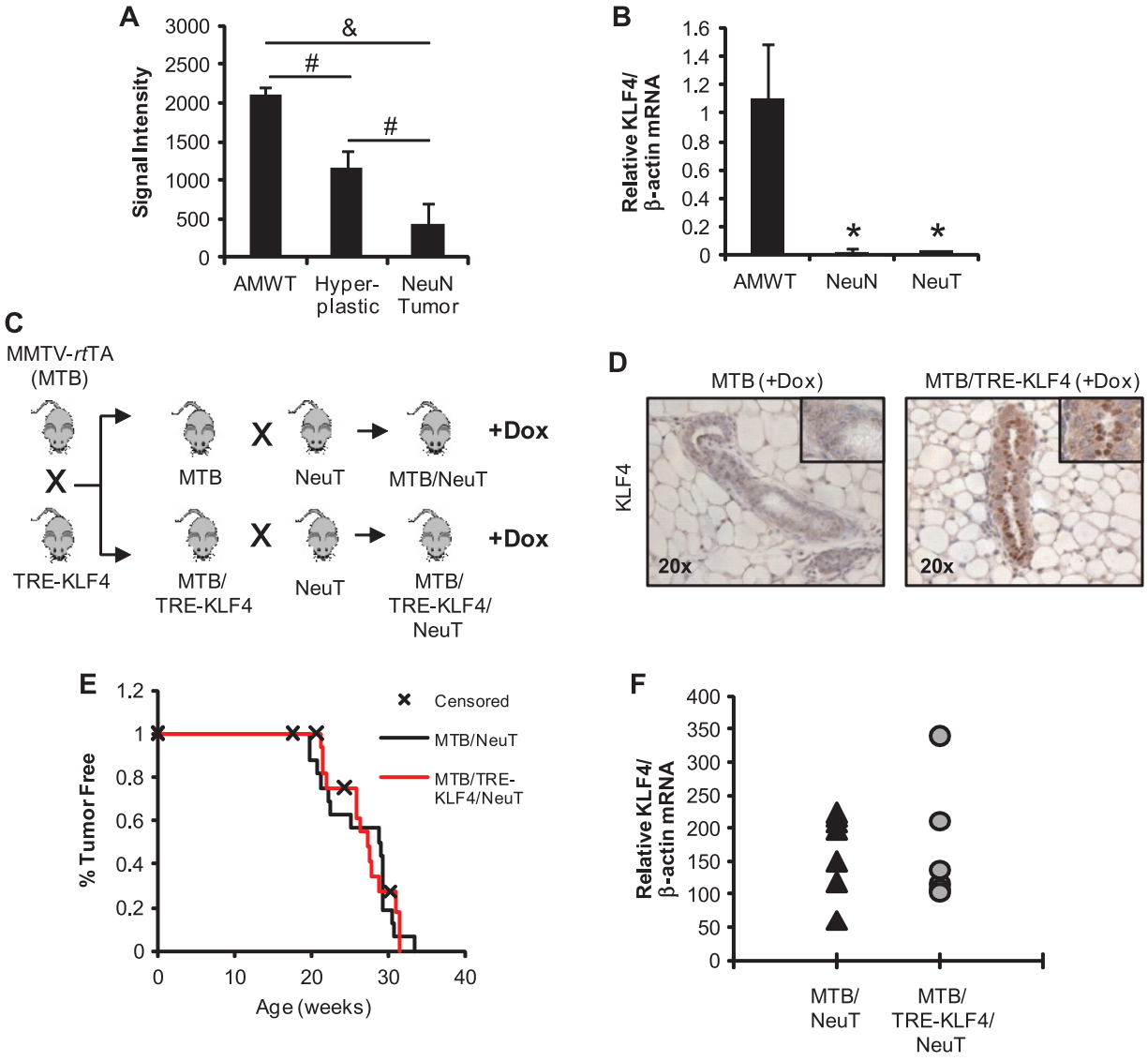


**Figure 5.** Loss of *KLF4* expression in HER2/*Neu*–induced mouse mammary tumors. (A) Microarray analysis of MMTV-*Neu*–induced tumors (NeuN) [26] reveals a significant reduction in KLF4 expression when compared with both age-matched wild-type (AMWT) and hyperplastic tissue. $^{\#}P < 1.0e - 4$. $^{\&}P < 2.0e - 7$. (B) qRT-PCR confirms decreased expression of *KLF4* in NeuN tumors compared with AMWT glands. The reduction in *KLF4* expression is comparable between tumors from NeuN mice and tumors from transgenic mice expressing activated *Neu* (NeuT). AMWT, $n = 3$; NeuN, $n = 4$; NeuT, $n = 3$. $^{*}P < .05$. (C) Breeding paradigm used to generate triple-transgenic mice expressing the mammary-specific, reversible tetracycline transactivator (*rt*TA) (MTB), activated *Neu* (NeuT), and inducible *KLF4* (TRE-KLF4). (D) Doxycycline induction of KLF4 in the mammary epithelium of MTB/TRE-KLF4 mice. Both MTB control and MTB/TRE-KFL4 mice were administered water containing 5% sucrose and 2 mg/ml doxycycline (Sigma) for 3 weeks before tissue harvest. Sections were stained with anti-KLF4. (E) NeuT-induced tumor latency is not altered in mice harboring the KLF4 expression cassette. All mice were administered doxycycline starting at weaning, and palpated twice weekly, starting at 15 weeks. MTB/NeuT, $n = 16$; MTB/NeuT/TRE-KLF4, $n = 14$. Statistical analysis and graphical representation of tumor development were generated by Kaplan-Meier survival analysis. Censored events or animals that were removed for reasons unrelated to tumor development are indicated with an "X." Median tumor latencies between groups were not different (log-rank test, $\chi^2 = 0.05$, $P = .81$). (F) Doxycycline-inducible *KLF4* expression is lost in tumors from the MTB/TRE-KLF4/NeuT mice. qRT-PCR showing comparable levels of *KLF4* expression between tumors that do not carry the inducible KLF4 transgene (MTB/NeuT, $n = 7$) and those that do (MTB/TRE-KLF4/NeuT, $n = 6$). $P = .98$.

into induced pluripotent stem (iPS) cells [38]. Therefore, we postulated that, in addition to E-cadherin, Snail may be a target through which KLF4 inhibits metastasis of breast cancer cells. We first analyzed Snail expression in 4T1 cells infected with control AdGFP or AdKLF4. KLF4 overexpression in 4T1 cells decreased both *Snail* transcript and protein (Figure 6, *A* and *B*) while at the same time increasing E-cadherin levels. Decreased Snail protein expression was observed as early as 1 day after infection with AdKLF4 (Figure 6, *C* and *D*). Conversely, we found that loss of KLF4 (shKLF4) in the nontransformed MCF-10A cells in which KLF4 expression has been stably reduced [15] (Figure 6*F*) resulted in increased *Snail* mRNA and protein levels (Figure 6, *E* and *F*), as well as other molecular changes consistent with EMT, including decreased E-cadherin, a P120 isoform switch, and an increase in the mesenchymal marker, vimentin, when compared with the control cells (shNS). To determine whether KLF4 could directly influence transcription of the *SNAI1* gene, we performed ChIP assays

using primers to detect binding at consensus KLF4 binding motifs identified in the −1500 to +260 bases of the human *SNAI1* promoter (Figure 6*G*). Direct binding of endogenous KLF4 to site K1 and K2 was detected in MCF-10A cells (Figure 6*H*). MDA-MB-231 breast cancer cells have low levels of endogenous KLF4 (Figure 1*A*), and KLF4 binding to the *SNAI1* promoter was undetectable in control AdGFP-infected cells. However, binding of KLF4 to both the K1 and K2 sites in AdKLF4 infected cells was readily detected (Figure 6*I*). These results indicate that one mechanism whereby KLF4 regulates the migratory and invasive capacity of breast cancer cells and, in turn, tumor metastasis may be through direct transcriptional suppression of *SNAI1* expression.

## Discussion

This study demonstrates that forced expression of KLF4 in the orthotopic 4T1 model inhibits primary tumor growth and metastases

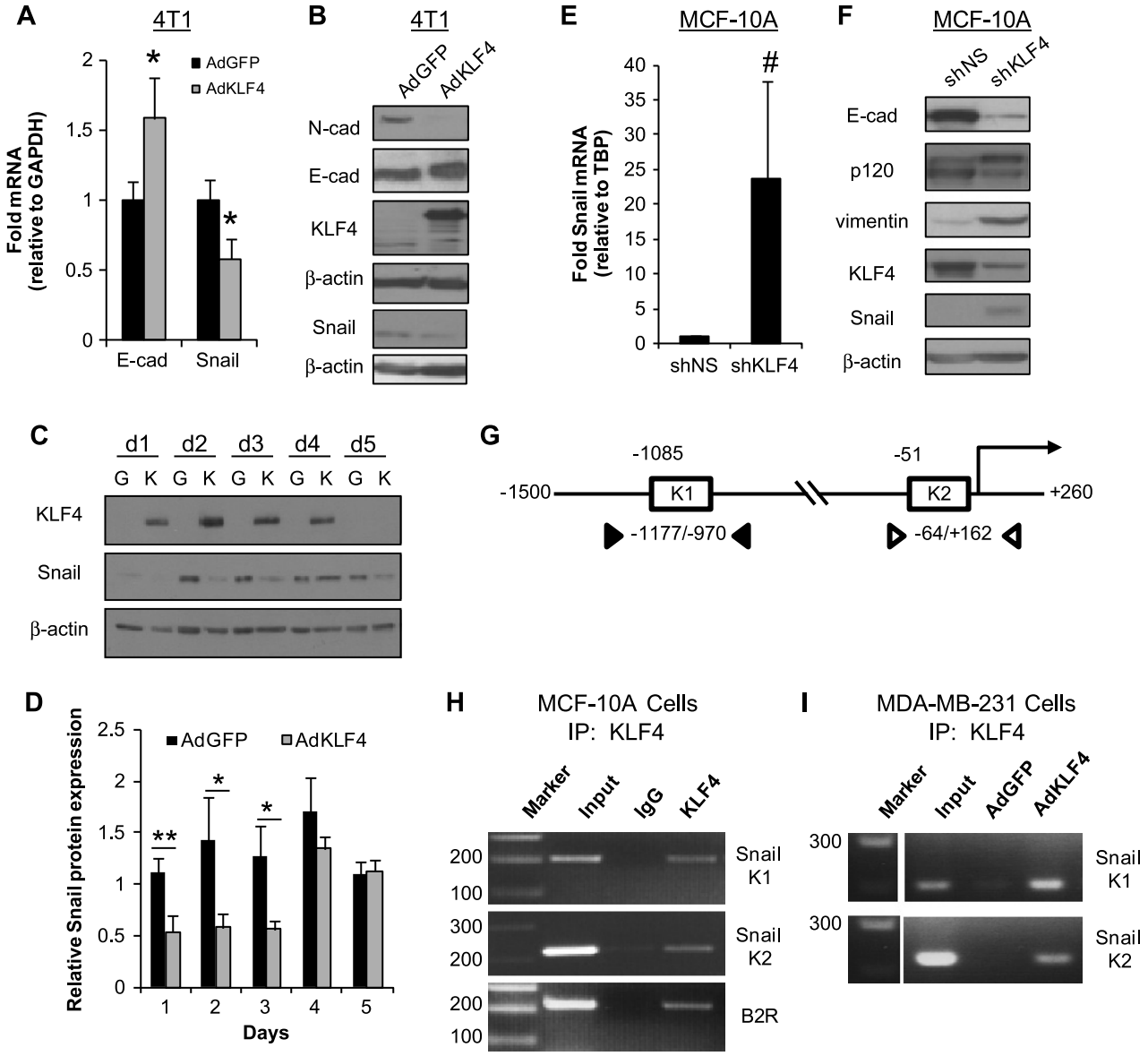


**Figure 6.** KLF4 inhibits Snail expression in mammary epithelial cells and tumor cells. (A and B) Forced expression of KLF4 (AdKLF4) in 4T1 cells inhibits Snail while increasing E-cadherin expression. (C) Representative Western blot analysis of Snail expression from day 1 (d1) to day 5 (d5) of adenoviral infection with either AdGFP (G) or AdKLF4 (K). (D) Densitometric quantification of Snail protein levels in AdGFP- and AdKLF4-infected 4T1 cells. (E) Stable knockdown of KLF4 (shKLF4) in MCF-10A cells results in increased *Snail* mRNA expression, compared with control (shNS) cells, as detected by qRT-PCR. (F) Representative Western blot showing increased Snail protein expression with loss of KLF4. Other changes associated with EMT are also observed such as increased vimentin and loss of E-cadherin. (G) The human *SNAI1* promoter contains two putative KFL4 binding sites as determined using MatInspector. Locations of primers flanking K1 and K2 sites are represented with arrowheads. (H and I) ChIP analysis was performed as indicated in Materials and Methods using MCF-10A cells or MDA-MB-231 cells infected with AdGFP or AdKLF4. Chromatin was immunoprecipitated with an antibody to KLF4. Immunoprecipitations with either IgG or anti-KLF4 in AdGFP-infected cells were used as negative controls. The bradykinin 2 receptor (B2R) promoter was used as a positive control. For RNA and protein, cells were plated in triplicate and harvested at 48 hours after infection in A and B or at day indicated in C and D. Experiments were performed at least three times. $^{*}P < .05$. $^{**}P < .01$. $^{\#}P < 2.0e-4$.

of breast cancer cells and that KLF4 expression is lost during HER2/*Neu*–induced mammary tumorigenesis. Taken together, these findings provide the first *in vivo* evidence supporting a role for KLF4 as a tumor and metastases suppressor in the breast. These functional data are further supported by the ability of KLF4 to inhibit breast cancer cell proliferation, promote apoptosis, and reduce expression of the metastasis inducer, Snail.

KLF4 has been recognized as a tumor suppressor in many types of cancer and, more recently, has been shown to suppress the migration and invasion of esophageal [39] and breast cancer [15] cells, suggesting its potential role as a metastasis suppressor. However, conflicting data exist regarding the function of KLF4 in breast cancer. Initial studies using immunohistochemical and *in situ* analyses of human breast tumors reported increased *KLF4* expression relative to adjacent, uninvolved epithelium [12], whereas subsequent reports have shown that *KLF4* mRNA levels are decreased in breast cancer, relative to normal tissue, and inversely correlated with increasing tumor grade [14]. *In vitro*, the ability of KLF4 to act as tumor suppressor or tumor promoter has been shown to be largely cell type specific [40], yet many studies examining the role of KLF4 in breast cancer have been conducted in fibroblast and other nonmammary epithelial cells. A recent study examining the role of KLF4 in the generation of iPS cells from fibroblasts *versus* epithelial cells [38] highlights the requirement of examining KLF4 in a cell-specific context. Even within the same breast cancer cell line, it has been reported that loss of KLF4 induces apoptosis in MCF7 cells [13], whereas others demonstrate that KLF4 inhibits estrogen-dependent proliferation in these same cells [14]. Recently, it has been reported that silencing of KLF4 in the MDA-MB-231 cells results in inhibition of primary tumor growth in immunocompromised nonobese diabetic/severe combined immunodeficiency mice [41]. These findings are in direct contrast to the reduced tumor volume seen with forced KLF4 expression in the immunocompetent 4T1 tumor model described here (Figure 4). Like the 4T1 cells, MDA-MB-231 cells have much lower levels of KLF4 compared with less aggressive breast cancer cell lines [15,42]. These data suggest that a basal level of KLF4 may be required for cell growth and survival, and this is consistent with data from MCF-10A cells where KLF4 suppression causes a decrease in proliferation [15].

Tumorigenic progression is a multistep process. Here we show, in concordance with human breast cancer arrays, that KLF4 expression is progressively suppressed in a mouse model of metastatic breast cancer that is induced by HER2/*Neu* overexpression (Figure 5*A*). Whereas KLF4 overexpression can be seen in the normal mammary epithelium of the MTB/TRE-KLF4 mice, the inability to exogenously express KLF4 in the HER2/*Neu*-induced tumors suggests that KLF4 precludes tumor formation. As the transgenic expression of NEU and KLF4 is not ubiquitous throughout the normal mammary epithelium, one possibility is that the cells expressing NEU, and not KLF4, are those capable of forming tumors. It is also is feasible that HER2/*Neu* signaling may posttranscriptionally block KLF4 expression.

Before the study reported herein, little was known regarding the role of KLF4, if any, during metastatic progression. Recently, Tian et al. [39] found that KLF4 was suppressed by microRNA (miR)-10b, resulting in increased migration and invasion of esophageal cancer cells. This becomes significant in the context of breast cancer as Ma et al. [43] have shown that silencing of miR-10b inhibits metastasis of 4T1 mouse tumors. We found that KLF4 expression inhibits lung and liver micrometastases in this same model (Table 1 and Figure 5). Other evidence supporting such a role for KLF4 is its ability to suppress EMT in mammary epithelial cells as well as support the mesenchymal-to-epithelial transition during iPS cell generation from fibroblasts [15,38]. Compelling evidence for EMT in breast cancer and its role in metastasis comes from recent studies examining a transcriptional driver of this process, Snail, and its elevated expression in carcinomas associated with lymph node metastases, as well as its expression in distal metastases [21,44,45]. We found that, in addition to other molecular changes associated with EMT, KLF4 suppresses Snail expression in both mammary epithelial cells and breast tumor cells (Figure 6). ChIP analyses indicate that this effect likely occurs through direct binding and regulation of the *SNAI1* gene by KLF4. The concurrent inhibition of Snail and activation of E-cadherin by KLF4 suggests that induction of mesenchymal-to-epithelial transition is one mechanism whereby KLF4 inhibits metastasis. KLF4 and Snail seem to act in a yin-and-yang manner to modulate the epithelial phenotype. Whereas KLF4 maintains the expression of a multitude of epithelial-specific genes [32], Snail transcriptionally suppresses several of the same targets. Of note, KLF4 is also repressed by Snail [46]. Thus, it seems that a negative feedback loop exists between these two transcriptional regulators to control the epithelial or mesenchymal states.

It has been well established that breast cancer is not one, biologically distinct disease, but a heterogeneous group of at least five different molecular subtypes, each having different outcomes [47,48]. While our findings begin to shed some light on the function of KLF4 in breast cancer and metastatic progression using two separate mouse models of mammary tumorigenesis, it is important that additional models, both *in vitro* and *in vivo*, representing various breast cancer subtypes be examined. Molecular heterogeneity of breast cancer could, in part, be explained by the stem cell hypothesis [49], whereby tumors arise from mammary stem cells that are endowed with multilineage differentiation potential and self-renewal [50]. As KLF4 plays a role in the induction and maintenance of iPS cells, it will also be important to determine whether similar regulation occurs in mammary and tumor stem cells. In summary, loss of KLF4 during mammary tumorigenesis, in combination with the ability of KLF4 to reduce tumor formation and metastasis, supports a tumor and metastasis-suppressive role for KLF4 in breast cancer. Whereas further studies are required to characterize the reciprocal regulation between Snail and KLF4, our findings provide new insights into a potential mechanism by which KLF4 inhibits breast cancer metastasis.

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## Supplemental Materials and Methods

### *Immunocytochemistry*

Cells were grown on glass coverslips and fixed in 4% paraformaldehyde containing 4% sucrose, permeabilized with 0.25% Triton X-100, blocked with 10% bovine serum albumin, and incubated with anti-Flag M2 (1:500) for 2 hours at 37°C. After incubation with Alexa Fluor 594–conjugated secondary antibody (Invitrogen, Molecular Probes), coverslips were mounted with Vectashield hardest mounting medium containing DAPI (Vector Laboratories, Burlingame, CA) and examined by fluorescence microscopy.

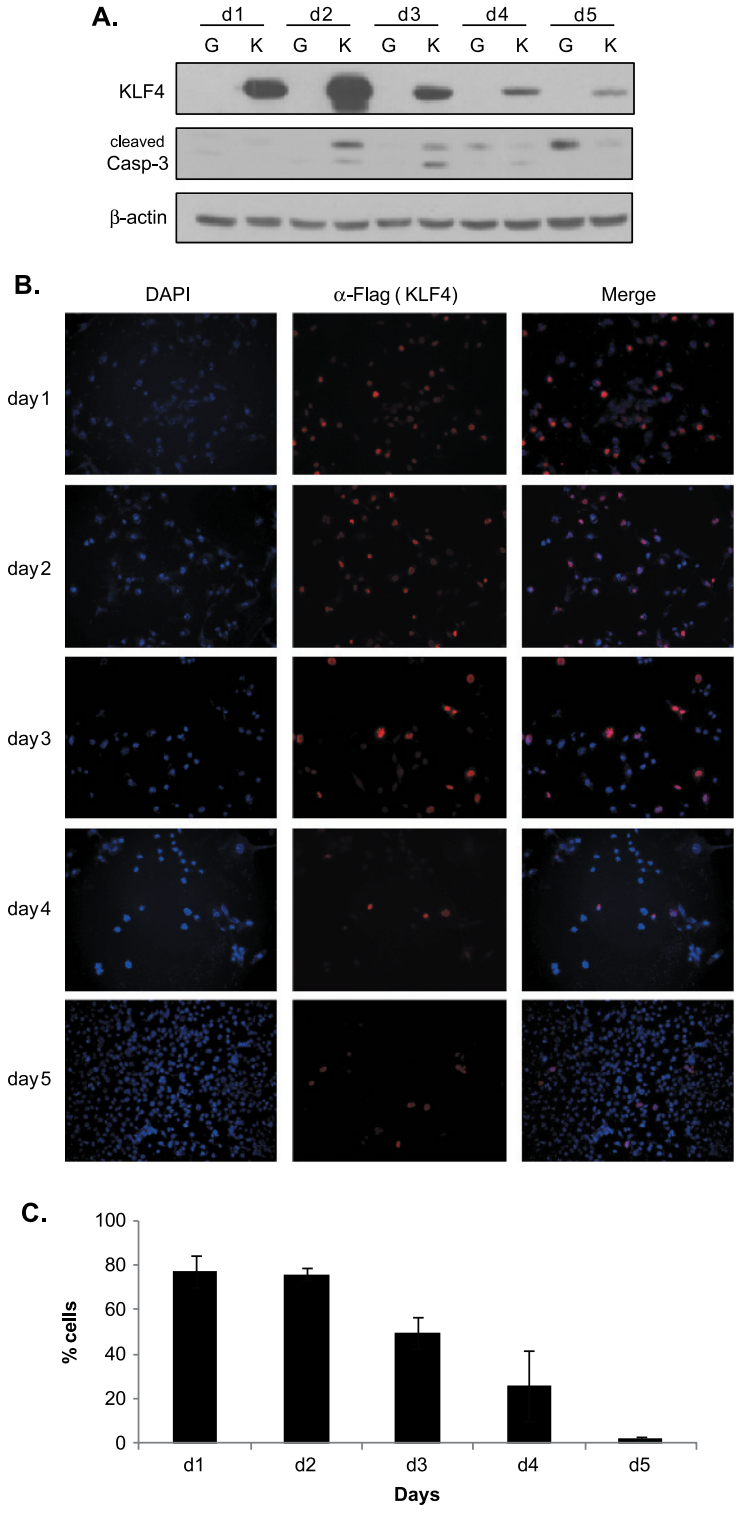


**Figure W1.** Time course of AdKLF4 overexpression in 4T1 cells. (A) Western blot analysis of AdGFP- (G) and AdKLF4- (K) infected 4T1 cells, *in vitro*. (B) Immunocytochemical detection of AdKLF4 expression during a 5-day time course in 4T1 cells. KLF4-overexpressing cells were labeled with anti-Flag (red). Nuclei were counterstained with DAPI (blue). (C) Bar graph representing percent of cells infected with AdKLF4. An average of two 20× fields/coverslip was counted for each time point. Experiments were performed in duplicate.